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PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) :	Barany et al.	)	Examiner: B. J. Forman
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For :	COUPLED POLYMERASE CHAIN REACTION-RESTRICTION ENDONUCLEASE DIGESTION-LIGASE DETECTION REACTION PROCESS	)	

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AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231
Box:

Dear Sir:

In response to the May 15, 2001, office action, please amend the above-identified patent application as follows:

In the Claims:

Please amend claims 1-14 and 17 as follows:

1. (Amended) A method for identifying one or more low abundance sequences differing by one or more single-base changes, insertions, or deletions from a high abundance sequence, in a sample containing a plurality of target nucleotide sequences comprising:

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providing a sample potentially containing one or more low abundance target nucleotide sequences with at least one sequence difference each from the high abundance target sequences;

providing a primary oligonucleotide primer set characterized by (a) a first oligonucleotide primer containing a target-specific portion, and (b) a second oligonucleotide primer containing a target-specific portion, wherein the primary oligonucleotide primers

hybridize to complementary strands of high and low abundance target nucleotide sequences to permit formation of a polymerase chain reaction product, but have a mismatch which interferes with formation of such a polymerase chain reaction product when hybridized to any other nucleotide sequence present in the sample;

providing a polymerase;

blending the sample, the primary oligonucleotide primers, and the polymerase to form a primary polymerase chain reaction mixture;

subjecting the primary polymerase chain reaction mixture to two or more polymerase chain reaction cycles;

providing a secondary oligonucleotide primer set characterized by (a) a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and (b) a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, wherein the secondary oligonucleotide primers in a particular set hybridize to complementary strands of the primary extension products to permit formation of a secondary polymerase chain reaction product which contains or creates a restriction endonuclease recognition site when amplifying the high abundance target, but does not contain or create a restriction endonuclease recognition site when amplifying the one or more low abundance targets;

providing a polymerase;

blending the primary extension products, the secondary oligonucleotide primers, and the polymerase to form a secondary polymerase chain reaction mixture;

subjecting the secondary polymerase chain reaction mixture to two or more polymerase chain reaction cycles, wherein high abundance secondary extension products contain a restriction site but low abundance secondary extension products do not;

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providing a restriction endonuclease;

blending the secondary extension product and the restriction endonuclease to form an endonuclease digestion reaction mixture;

subjecting the endonuclease digestion reaction mixture to an endonuclease digestion reaction such that the restriction endonuclease recognizes and cleaves the restriction endonuclease recognition site contained within or created when amplifying the high abundance target but not the low abundance target in the secondary extension products, thus selectively destroying the high abundance secondary extension products;

providing a tertiary oligonucleotide primer set characterized by (a) a first tertiary primer containing the same sequence as the 5' upstream portion of the first oligonucleotide primer of the secondary oligonucleotide primer set, and (b) a second tertiary primer containing the same sequence as the 5' upstream portion of a second oligonucleotide primer of the secondary oligonucleotide primer set, wherein the set of tertiary oligonucleotide primers are amplification primers for amplification of all the secondary extension products;

blending the secondary extension products, the tertiary oligonucleotide primer set, and the polymerase to form a tertiary polymerase chain reaction mixture;

subjecting the tertiary polymerase chain reaction mixture to two or more polymerase chain reaction cycles;

providing a plurality of oligonucleotide probe sets, each set characterized by (a) a first oligonucleotide probe, having a tertiary extension product-specific portion and a detectable reporter label, and (b) a second oligonucleotide probe, having a tertiary extension product-specific portion, wherein the oligonucleotide probes in a particular set ligate together when hybridized adjacent to one another on a complementary tertiary extension product-specific portion, but have a mismatch which interferes with said ligation when hybridized to any other nucleotide sequence present in the sample;

providing a ligase;

blending the tertiary extension product, the plurality of oligonucleotide probe sets, and the ligase to form a ligase detection reaction mixture;

subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles comprising a denaturation treatment, wherein any hybridized oligonucleotides are separated from the tertiary extension products, and a hybridization treatment, wherein the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to their respective tertiary extension products, if present, and ligate to one another to form a ligation product sequence containing (a) the detectable reporter label and (b) the tertiary extension product-specific portions connected together, wherein the oligonucleotide probe sets may hybridize to nucleotide sequences other than their respective complementary tertiary extension products but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment; and

detecting the reporter labels of the ligation product sequences, thereby identifying the presence of one or more low abundance target nucleotide sequences in the sample.

2. (Amended) A method according to claim 1, wherein the oligonucleotide probes in an oligonucleotide probe set have a unique length whereby the ligation product sequences which they form are distinguished from other ligation product sequences, said method comprising:

separating the ligation product sequences by electrophoretic mobility prior to said detecting and

distinguishing, after said detecting, the ligation product sequences which differ in electrophoretic mobility.

3. (Amended) A method according to claim 1, wherein the second oligonucleotide probe of each oligonucleotide probe set further comprises an addressable array-specific portion, said method further comprising:

providing a solid support comprising an array of address-specific capture oligonucleotides, and

contacting the ligase detection reaction mixture, after said subjecting it to one or more ligase detection reaction cycles, with the solid support to hybridize the ligation product sequences to the capture oligonucleotides in a base-specific manner, thereby capturing the addressable array-specific portions to the solid support at the site with the complementary capture oligonucleotide, wherein said detecting identifies the presence of ligation product sequences captured using the addressable array-specific portions and immobilized to the solid support at particular sites, thereby identifying the presence of one or more target nucleotide sequences in the sample.

4. (Amended) A method according to claim 1 further comprising:

A1 quantifying the amount of the low abundance sequence, wherein said quantifying comprises:

providing a known amount of one or more marker target nucleotide sequences as an internal standard;

providing one or more internal standard sequence-specific oligonucleotide probe sets specifically designed for hybridization to the internal standard, wherein the internal standard sequence-specific oligonucleotide probe sets have (1) a first oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence,

and (2) a second oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence and a detectable reporter label;

blending the internal standard, and the internal standard sequence-specific probe sets, with the ligase detection reaction mixture; and

quantifying the amount of ligation product sequences by comparing the amount of ligation product sequences generated from the unknown low abundance sample to the amount of ligation product sequences generated from said internal standard to provide a quantitative measure of one or more low abundance target nucleotide sequences in the sample.

5. (Amended) A method according to claim 4, wherein one or more low abundance sequence is present in less than a 1:1,000 molar ratio relative to the amount of the high abundance sequence present in the sample.

6. (Amended) A method according to claim 4, wherein one or more low abundance sequence is present in less than a 1:10,000 molar ratio relative to the amount of the high abundance sequence present in the sample.

7. (Amended) A method according to claim 4, wherein one or more low abundance sequence is present in less than a 1:100,000 molar ratio relative to the amount of the high abundance sequence present in the sample.

8. (Amended) A method according to claim 1, wherein prior to providing the secondary oligonucleotide primer set, said method comprises:

A1 providing a pre-secondary oligonucleotide primer set characterized by (a) a first oligonucleotide primer, having a target-specific portion, and (b) a second oligonucleotide primer, having a target-specific portion, wherein the target-specific portions are identical or substantially identical to the secondary oligonucleotide primer set but at least one primer contains one or more nucleotide analogs, wherein the oligonucleotide primers in a particular pre-secondary oligonucleotide primer set hybridize to complementary strands of the primary extension products to form a pre-secondary polymerase chain reaction product which contains one or more nucleotide analogs and opposite strand base changes, wherein the pre-secondary oligonucleotide primer set facilitates conversion of the primary polymerase chain

reaction product sequence into a restriction endonuclease recognition site in the subsequent secondary polymerase chain reaction;

providing a polymerase;

blending the primary extension products, the pre-secondary oligonucleotide primers, and the polymerase to form a pre-secondary polymerase chain reaction mixture;

subjecting the pre-secondary polymerase chain reaction mixture to two or more polymerase chain reaction cycles comprising a denaturation treatment, wherein hybridized nucleic acid sequences are separated, a hybridization treatment, wherein the secondary oligonucleotide primers hybridize to the primary extension products, an extension treatment, wherein the hybridized secondary oligonucleotide primers are extended to form pre-secondary extension products complementary to the primary extension products, wherein the pre-secondary extension products contain one or more nucleotide analogues and opposite strand base changes which facilitate conversion of the primary polymerase chain reaction product sequence into a restriction endonuclease recognition site in the subsequent secondary polymerase chain reaction, wherein the pre-secondary extension products are then used in place of the primary extension products in the secondary polymerase chain reaction mixture, whereby the efficiency and accuracy of converting the high abundance primary polymerase chain reaction product into a secondary polymerase chain reaction product containing a restriction endonuclease site is improved.

9. (Amended) A method according to claim 8, wherein the oligonucleotide probes in an oligonucleotide probe set have a unique length whereby the ligation product sequences which they form are distinguished from other ligation product sequences, said method further comprising:

separating the ligation product sequences by electrophoretic mobility prior to said detecting and

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distinguishing, after said detecting, the ligation product sequences which differ in electrophoretic mobility.

10. (Amended) A method according to claim 8, wherein the second oligonucleotide probe of each set further comprises an addressable array-specific portion, said method further comprising:

providing a solid support comprising an array of address-specific capture oligonucleotides and

contacting the ligase detection reaction mixture, after said subjecting it to one or more ligase detection reaction cycles, with the solid support to hybridize the ligation product sequences to the capture oligonucleotides in a base-specific manner, thereby capturing the addressable array-specific portions to the solid support at the site with the complementary capture oligonucleotide, wherein said detecting identifies the presence of ligation product sequences captured using the addressable array-specific portions and immobilized to the solid support at particular sites, thereby identifying the presence of one or more target nucleotide sequences in the sample.

11. (Amended) A method according to claim 8 further comprising:

quantifying the amount of low abundance sequence, wherein said quantifying comprises:

providing a known amount of one or more marker target nucleotide sequences as an internal standard;

providing one or more internal standard sequence-specific oligonucleotide probe sets specifically designed for hybridization to the internal standard, wherein the internal standard sequence-specific oligonucleotide probe sets have (1) a first oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence, and (2) a second oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence and a detectable reporter label;

blending the internal standard and the internal standard sequence-specific probe sets with the ligase detection reaction mixture; and

A1 quantifying the amount of ligation product sequences by comparing the amount of ligation product sequences generated from the unknown low abundance sample to the amount of ligation product sequences generated from said internal standard to provide a quantitative measure of one or more low abundance target nucleotide sequences in the sample.

12. (Amended) A method according to claim 11, wherein one or more of a low abundance sequence is present in a molar ratio of than less than 1:1,000 relative to the amount of the high abundance sequence in the sample.

13. (Amended) A method according to claim 11, wherein one or more of a low abundance sequence is present in a molar ratio of than less than 1:10,000 relative to the amount of the high abundance sequence in the sample.

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14. (Amended) A method according to claim 11, wherein one or more of a low abundance sequence is present in a molar ratio of than less than 1:100,000 relative to the amount of the high abundance sequence in the sample.

17. (Amended) A method according to claim 1 further comprising:

blending the ligation product sequences and the restriction endonuclease, wherein the restriction endonuclease recognizes and cleaves the restriction endonuclease recognition site contained within any remaining high abundance target, thereby selectively destroying the high abundance tertiary extension products.

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REMARKS

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested. Pursuant to 37 CFR § 1.21, attached as Appendix A is a Version With Markings to Show Changes Made.

Cancer Detection

As the second leading cause of death in this country, almost 600,000 people will die from cancer per year making cancer one of the most alarming of all medical diagnosis. Lifetime risks for developing invasive cancers in men and women are 50 percent and 33 percent, respectively. Expectations are that more than 1.2 million new cases of cancer will be diagnosed in the United States in 1995. Healthcare expenses for cancer in 1994 were approximately $104 billion. However, the full impact of cancer on families and society is not measured only by the amount of money spent on its diagnosis and treatment. A significant number of people are stricken with cancer in their most productive years. Cancers accounted for 18 percent of premature deaths in 1985 and in 1991 more than 9,200 women in the U.S. died from breast cancer before the age of 55.

Currently, diagnosis of cancer is based on histological evaluation of tumor tissue by a pathologist. After a cancer is diagnosed, treatment is determined primarily by the extent or stage of the tumor. Tumor stage is defined by clinical, radiological, and laboratory methods. Standardized classification systems for the staging of tumors have been developed to clearly convey clinical information about cancer patients. Staging provides important prognostic information and forms the basis of clinical studies which allow the testing of new treatment strategies. A staging system was developed (TNM staging system), which classifies tumors according to the size of the primary tumor, the number of regional lymph nodes in which cancer is found, and the presence or absence of metastases to other parts of the body. Smaller cancers with no affected lymph nodes and no distant metastases are considered early stage cancers, which are often amenable to cure through surgical resection. A common measure of prognosis is the 5-year survival rate (i.e., the proportion of patients alive five years after the diagnosis of a cancer at a given stage). While 5-year survival rates for many cancers have improved over the last few decades, the fact that some early stage cancers recur within five years or later has led researchers to explore other additional

prognostic markers including histological grade, cytometry results, hormone receptor status, and many other tumor markers. Most recently, investigators have explored the use of molecular alterations in cancers as prognostic indicators.

Genetic alterations found in cancers, such as point mutations and small deletions can act as markers of malignant cells.

Detection of Minority Nucleic Acid Sequences

A number of procedures have been disclosed to detect cancer using polymerase chain reaction ("PCR"). Sidransky, et al., "Identification of *ras* Oncogene Mutations in the Stool of Patients with Curable Colorectal Tumors," Science 256: 102-05 (1992) detects colon cancer by identification of K-*ras* mutations. This involves a PCR amplification of total DNA, cloning into a phage vector, plating out the phage, repeated probing with individual oligonucleotides specific to several different K-*ras* mutations, and counting the percentage of positive plaques on a given plate. This is a technically difficult procedure which takes three days to complete, whereby the ratio of mutant to wild-type DNA in the stool sample is determined. Brennan, et al., "Molecular Assessment of Histopathological Staging in Squamous-Cell Carcinoma of the Head and Neck," N. Engl. J. Med. 332(7): 429-35 (1995), finds p53 mutations by sequencing. This specific mutation is then probed for in margin tissue using PCR amplification of total DNA, cloning into a phage vector, plating out the phage, probing with an individual oligonucleotide specific to the mutation found by sequencing, and counting the percentage of positive plaques on a given plate. Berthelemy, et al., "Brief Communications--Identification of K-*ras* Mutations in Pancreatic Juice in the Early Diagnosis of Pancreatic Cancer," Ann. Int. Med. 123(3): 188-91 (1995) uses a PCR/restriction enzyme process to detect K-*ras* mutations in pancreatic secretions. This technique is deficient, however, in that mutations are not quantified. Similarly, Tada, et al., "Detection of *ras* Gene Mutations in Pancreatic Juice and Peripheral Blood of Patients with Pancreatic Adenocarcinoma," Cancer Res. 53: 2472-74 (1993) and Tada, et al., "Clinical Application of *ras* Gene Mutation for Diagnosis of Pancreatic Adenocarcinoma," Gastroent. 100: 233-38 (1991) subject such samples to allele-specific PCR to detect pancreatic cancer. This has the disadvantages of providing false positives due to polymerase extension off normal template, requiring electrophoretic separation of products to distinguish from primer dimers, being unable to multiplex closely-clustered sites due to interference of overlapping primers, being unable to detect single base or small insertions and

deletions in small repeat sequences, and not being practically suitable for quantification of mutant DNA in a high background of normal DNA. Hayashi, et al., "Genetic Detection Identifies Occult Lymph Node Metastases Undetectable by the Histopathological Method," Cancer Res. 54: 3853-56 (1994) uses an allele-specific PCR technique to find K-*ras* or p53 mutations to identify occult lymph node metastases in colon cancers. A sensitivity of one tumor cell in one thousand of normal cells is claimed; however, obtaining quantitative values requires laborious cloning, plating, and probing procedures. In Mitsudomi, et al., "Mutations of *ras* Genes Distinguish a Subset of Non-small-cell Lung Cancer Cell Lines from Small-cell Lung Cancer Cell Lines," Oncogene 6: 1353-62 (1991), human lung cancer cell lines are screened for point mutations of the K-, H-, and N-*ras* genes using restriction fragment length polymorphisms created through mismatched primers during PCR amplification of genomic DNA. The disadvantages of such primer-mediated RFLP include the requirement of electrophoretic separation to distinguish mutant from normal DNA, limited applicability to sites that may be converted into a restriction site, the requirement for additional analysis to determine the nature of the mutation, and the difficulty in quantifying mutant DNA in a high background of normal DNA. Further, these procedures tend to be laborious and inaccurate.

Coupled PCR/ligation processes have been used for detection of minority nucleotide sequences in the presence of majority nucleotide sequences. A PCR/LDR process is used by one group to detect HIV mutants. This assay, however, cannot be used for multiplex detection.

Colorectal lesions have been detected by a process involving PCR amplification followed by an oligonucleotide ligation assay. This process was developed as an advance over Powell, et al., "Molecular Diagnosis of Familial Adenomatous Polyposis," N. Engl. J. Med. 329(27): 1982-87 (1993). These techniques tend to be limited and difficult to carry out.

Other procedures have been developed to detect minority nucleotide sequences, including virus revertants, by PCR and restriction enzyme cleavage. The disadvantages of this method (known as MAPREC) include the requirement for electrophoretic separation to distinguish mutant from normal DNA, limited applicability to sites that may be converted into a restriction site, the requirement for additional analysis to determine the nature of the mutation, and difficulty in quantifying mutant DNA in a high background of normal DNA. In Kuppuswamy, et al., "Single Nucleotide Primer Extension to Detect Genetic Diseases: Experimental Application to Hemophilia G (Factor IX) and Cystic

Fibrosis Genes," Proc. Natl. Acad. Sci. USA 88: 1143-47 (1991), a PCR process is carried out using two reaction mixtures for each fragment to be amplified with one mixture containing a primer and a labeled nucleotide corresponding to the normal coding sequence, while the other mixture contains a primer and a labeled nucleotide corresponding to the mutant sequence. The disadvantages of such mini sequencing (i.e., SNuPe) are that the mutations must be known, it is not possible to multiplex closely clustered sites due to interference of overlapping primers, it is not possible to detect single base or small insertions and deletions in small repeat sequences, and four separate reactions are required. A mutagenically separated PCR process has been used to distinguish normal and mutant alleles, using different length allele-specific primers. The disadvantages of MS-PCR include possibly providing false positives due to polymerase extension off normal template, requiring electrophoretic separation of products to distinguish from primer dimers, the inability to multiplex closely-clustered sites due to interference of overlapping primers, the inability to detect single base or small insertions and deletions in small repeat sequences, and not being ideally suited for quantification of mutant DNA in high background of normal DNA. In one process, mutations are detected in a process having a PCR phase followed by a phase involving single strand conformation polymorphism ("SSCP") of the amplified DNA fragments. The disadvantages of SSCP include the requirement for electrophoretic separation to distinguish mutant conformer from normal conformer, the failure to detect 30% of possible mutations, the requirement for additional analysis to determine the nature of the mutation, and the inability to distinguish mutant from silent polymorphisms.

Nucleotide Conversion Fidelity

Many of the approaches to detecting the presence of a given sequence or sequences in a polynucleotide sample involve amplification of the minority sequence(s) by polymerase chain reaction ("PCR"). In this method, primers complementary to opposite end portions of the selected sequence(s) are used to promote, in conjunction with thermal cycling, successive rounds of primer-initiated replication. The amplified sequence(s) may be readily identified by a variety of techniques. This approach is particularly useful for detecting the presence of low-copy sequences in a polynucleotide-containing sample, e.g., for detecting pathogen sequences in a body-fluid sample. However, a nonselective PCR strategy will amplify both mutant and wild-type alleles with approximately equal efficiency, resulting in low abundance mutant alleles comprising only a small fraction of the final product. If the mutant sequence

comprises less than 25% of the amplified product, it is unlikely that DNA sequencing will be able to detect the presence of such an allele. Although it is possible to accurately quantify low abundance mutations by first separating the PCR products by cloning and subsequently probing the clones with allele-specific oligonucleotides ("ASOs"), this approach is time consuming. In contrast, allele-specific PCR methods can rapidly and preferentially amplify mutant alleles. For example, multiple mismatch primers have been used to detect H-*ras* mutations at a sensitivity of 1 mutant in 10^5 wild-type alleles and claims as high as 1 mutant in 10^6 wild-type alleles have been reported. However, careful evaluation suggests these successes are limited to allele-specific primers discriminating through 3' purine-purine mismatches. For the more common transition mutations, the discriminating mismatch on the 3' primer end (i.e., G:T or C:A mismatch) will be removed in a small fraction of products by polymerase error during extension from the opposite primer on wild-type DNA. Thereafter, these error products are efficiently amplified and generate false-positive signal. One strategy to eliminate this polymerase error problem is to deplete wild-type DNA early in PCR.

Several investigators have explored selective removal of wild-type DNA by restriction endonuclease ("RE") digestion in order to enrich for low abundance mutant sequences. These RFLP methods detect approximately 1 mutant in 10^6 wild-type or better by combining the sensitivity of polymerase with the specificity of restriction endonucleases. One approach has used digestion of genomic DNA followed by PCR amplification of the uncut fragments (RFLP-PCR) to detect very low-level mutations within restriction sites in the H-*ras* and p53 genes. Similar results have been obtained by digestion following PCR and subsequent amplification of the uncleaved DNA now enriched for mutant alleles (PCR-RFLP) Although sensitive and rapid, RFLP detection methods are limited by the requirement that the location of the mutations must coincide with restriction endonuclease recognition sequences. To circumvent this limitation, primers that introduce a new restriction site have been employed in "primer-mediated" RFLP. However, subsequent investigators have demonstrated that errors are produced at the very next base by polymerase extension from primers having 3' natural base mismatches. Such templates fail to cleave during restriction digestion and amplify as false-positives that are indistinguishable from true positive products extended from mutant templates.

Use of nucleotide analogs may reduce errors resulting from polymerase extension and improve base conversion fidelity. Nucleotide analogs that are designed to base-pair with more than one of the four natural bases are termed "convertides." Base

incorporation opposite different convertides has been tested. For each analog, PCR products were generated using *Taq* polymerase and primers containing an internal nucleotide analog. The products generated showed a characteristic distribution of the four bases incorporated opposite the analogs. Of significance, these products retained the original sequence at all natural base positions. Convertides readily form degenerate amplification products by virtue of their ability to assume different hydrogen bonding patterns through tautomeric shift. Thus, PCR primers containing convertides may be used to facilitate base conversion. In principle, using the 6H,8H-3,4-dihydropyrimido[4,5-c][1,2]oxazine-7-one analog Q_6), which is known to exhibit both the C-like and T-like tautomeric forms at the 3' end of the primer, a C-G base-pair may be converted to a T-A base pair. Due to the better geometry, DNA polymerases may "read," or extend better, from a Q_6-G pair than a T-G mismatch (wobble base pair). Similarly, DNA polymerases may "write," or incorporate both G and A bases opposite Q_6, whereas A is always inserted opposite a T base. Thus, the Q_6 analog primer serves as an intermediary, providing a "preconversion" step before a natural base primer is added to selectively amplify the desired product from the degenerate pool. While nucleotide analogs have great potential, they have not been tested in high sensitivity assays. There is a need for a method that optimizes the fidelity of the analog conversion in the PCR process.

Optimization of PCR/RE/LDR

As discussed above, PCR used with a high fidelity conversion process would provide a valuable method for the amplification of mutant gene sequences. By designing primers with one or more mismatches, mutant DNA template can be efficiently extended, while poor extension is achieved on wild-type DNA template. However, once these primers extend with or without a mismatch, the products thereafter are perfect matches for the primer in subsequent PCR cycles. Thus, false positive signals are amplified in subsequent cycles. Moreover, PCR error can generate a base change in the template, which perfectly matches the primer. AS-PCR can detect pyrimidine↔purine transversions at sensitivities of 1 in 10^5. Nevertheless, the majority of cancer-associated mutations are C↔T and A↔G transitions, as, for example, are over 80% of p53 point mutations. A DNA diagnostic method is needed to accurately quantify this type of low abundance mutation.

The ligation detection reaction ("LDR") in conjunction with PCR has been used to quantify small amounts of PCR extension product. LDR uses two adjacent primers and a thermostable ligase to distinguish all four bases potentially found at any position in a

DNA sequence. Thermostable ligase demonstrates the highest fidelity when the discriminating base is located at the 3' end of the upstream primer. PCR/LDR (i.e., PCR of a sequence from genomic DNA followed by LDR) can detect mutations with a sensitivity of approximately 1 mutant allele in 4,000 normal alleles. Sensitivity of approximately 1 in 10^6 has been achieved by combining PCR with restriction endonuclease digestion of wild-type DNA. Mutations occurring within the restriction site prevent cleavage of the mutant allele, while wild-type alleles bearing canonical restriction site sequence are depleted. As a result, subsequent PCR cycles preferentially amplify mutant DNA. If a mutation site is not within an endonuclease recognition site present in wild-type DNA, a restriction site must be introduced. This is typically done by PCR using a primer or primers with mismatched bases. Mutations cannot be detected in any portion of the restriction site spanned by the primers, since those bases are introduced directly through the primers. In a random DNA sequence, over 20% of bases are contained within a preexisting four-base restriction site and 60% of bases are within a four-base subsequence that can be converted into a restriction site by a single base change. In these small sites, 3' terminal base mismatch primers must frequently be used. While conceptually straightforward, 3' mismatch extension has proven to be difficult. The introduction of interrupted palindromic restriction sites has been more successful using internal mismatch primers spanning one half-site through the intervening bases up to the other half-site. Several perfectly matched bases stabilize the 3' end of the mismatch primer. However, this approach may be used only if the second half-site is present naturally in wild-type DNA. Mutations in the second half-site prevent digestion. Only mutations occurring at bases within the recognition sequence are detectable by RFLP methods. Mutations occurring at bases outside a preexisting restriction site in wild-type DNA may be detected by introducing a new restriction site containing that base.

Restriction endonucleases recognizing interrupted palindromes are less abundant than endonucleases recognizing contiguous four- and six-base sites. Multiple base changes would often be required to introduce an interrupted palindrome restriction site to identify mutations at any base.

More recently, methods of identifying known target sequences by probe ligation methods have been reported. In one approach, known as oligonucleotide ligation assay ("OLA"), two probes or probe elements, that span a target region of interest, are hybridized to the target region. Where the probe elements base pair with adjacent target bases, the confronting ends of the probe elements can be joined by ligation, e.g., by treatment

with ligase. The ligated probe element is then assayed, evidencing the presence of the target sequence.

In a modification of this approach, the ligated probe elements act as a template for a pair of complementary probe elements. With continued cycles of denaturation, hybridization, and ligation in the presence of pairs of probe elements, the target sequence is amplified linearly, allowing very small amounts of target sequence to be detected and/or amplified. This approach is referred to as ligase detection reaction. When two complementary pairs of probe elements are utilized, the process is referred to as the ligase chain reaction, which achieves exponential amplification of target sequences. Techniques such as PCR/LDR that rely on mutant enrichment require optimization of reaction conditions in order to minimize random PCR errors. These errors would be indistinguishable from mutations originally present in clinical samples. One source of error-minimization may be found in optimization of the buffer conditions for PCR. Standard PCR buffers contain Tris, however the pK_a of Tris is strongly dependent on temperature. A PCR reaction containing Tris pH 8.3 (measured at 23°C) is approximately pH 7 near 65°C (the extension temperature), and drops to approximately pH 6 near 95°C (the template melting temperature). PCR error can result from template degradation and polymerase misincorporation. Template degradation occurs during periods of high temperature and low pH in each PCR cycle and limits product size in "long" PCR. Raising the buffer pH in long PCR (using Tris pH 9.1) reduces the amount of template cleavage and increases PCR efficiency. Although the efficiency of long PCR increases with higher pH, the level of mutations within these PCR products may also increase since high pH decreases the fidelity of *Taq* and *Pfu* polymerases. Use of alternative PCR buffers with lower $|\Delta pKa|$ can improve polymerase fidelity and still reduce template damage by maintaining more neutral pH over a wider temperature range. The addition of glycerol or formamide may reduce mutations arising from template damage during PCR cycling and may help avoid misextension from mispaired primers.

Thus, there is a need to improve buffer reaction conditions currently used in PCR, in order to minimize the opportunity for mismatches caused by PCR error. Increased analog conversion fidelity, alone, will not solve the need for an improved method of mutant DNA detection. In addition, there is a need to optimize PCR reaction conditions to decrease random PCR error, and finally, a method is needed that provides sensitive detection for the PCR extension products.

The rejection of claims 1-17 under 35 U.S.C. § 112 (2^{nd} para.) for indefiniteness is traversed in light of the above amendments except as set forth below. The amendments to claims 1-17 are made to correct minor informalities noted by the Patent and Trademark Office ("PTO") and have no affect on the scope of the claims.

Applicants submit that the meaning of the claimed "subjecting" steps in claim 1 is completely clear to one of ordinary skill in the art when reading these steps in the context of the remainder of the claim. For example, with regard to the "subjecting the primary polymerase chain reaction mixture to two or more . . ." limitation, the preceding steps explain what forms the primary PCR mixture, which is then subjected to PCR. The PCR procedure is well-known, so one of ordinary skill in the art would fully understand what happens to the primary PCR mixture during this step. With regard to the "subjecting the secondary polymerase chain reaction mixture to two or more . . ." limitation, the preceding steps explain what forms the secondary PCR mixture, which is then subjected to PCR. Again, the PCR procedure is well-known, so what happens to the secondary PCR mixture during this step would be fully understood to one of ordinary skill in the art. Likewise, as to the recitation of "subjecting the endonuclease digestion reaction mixture to . . .", the preceding steps explain what is present in the endonuclease digestion reaction mixture, which is then subjected to an enzyme digestion. The endonuclease restriction digestion procedure is well-known, so one skilled in the art would fully understand what happens to the endonuclease restriction digestion mixture during this step. With regard to the "subjecting the tertiary polymerase chain reaction mixture to two or more . . ." limitation, the preceding steps explain what forms the tertiary PCR reaction mixture, which is then subjected to PCR. Again, what happens to the tertiary PCR mixture during this step would be fully understood to those skilled in the art. Finally, for the recitation of "subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles . . .", the preceding steps explain what forms the ligase detection reaction mixture. The ligase detection reaction procedure is well-known to those skilled in the art, so what happens to the ligase detection reaction mixture during this step would be fully understood.

For all the above reasons, the rejection of claims 1-17 under 35 U.S.C. § 112 (2^{nd} para.) should be withdrawn.

The rejection of claims 1, 3-7, and 17, under 35 U.S.C. § 103(a) for obviousness over WO 97/31256 to Barany et al. ("Barany") in view of Jacobson et al., "A Highly Sensitive Assay For Mutant *ras* Genes And Its Application To The Study of

Presentation and Relapse Genotypes In Acute Leukemia," Oncogene, 9:553-563 (1994)("Jacobson") is respectfully traversed.

Barany teaches a method for identifying one or more of a plurality of sequences differing by one or more single base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. The method includes a ligation phase, a capture phase, and a detection phase. The ligation phase utilizes a ligation detection reaction ("LDR") between one oligonucleotide probe, having a target sequence-specific portion, and a detectable label. After the ligation phase, the capture phase is carried out by hybridizing the ligated oligonucleotide probes to a solid support with an array of immobilized capture oligonucleotides at least some of which are complementary to the addressable array-specific portion. Following completion of the capture phase, a detection phase is carried out to detect the labels of ligated oligonucleotide probes hybridized to the solid support. The ligation phase can be preceded by an amplification process.

Nowhere does Barany suggest using the claimed steps of "subjecting the primary polymerase chain reaction mixture to two or more polymerase chain reaction cycles" (let alone the prior steps of forming that mixture or providing the mixture's components), "subjecting the secondary polymerase chain reaction mixture to two or more polymerase chain reaction cycles" (let alone the prior steps of forming that mixture or providing the mixture's components), or "subjecting the endonuclease digestion reaction mixture to an endonuclease digestion reaction" (let alone the prior steps of forming that mixture or providing the mixture's components.)

Jacobson teaches a method for the detection of mutant *ras* genes and its application to the study of presentation and relapse genotypes in acute leukemia. The starting sample is PCR amplified with "matched primers" that amplify the entire N-*ras* codon 61, the known site of *ras* mutations. A secondary PCR procedure is carried out with mismatched "screening" primers to create a restriction endonuclease ("RE") site in the secondary extension products. The formation of the restriction site is dependent upon both introduced substitution and the normal codon 61 sequence. Therefore, only normal alleles contain the introduced restriction site. The secondary extension products are digested with an enzyme recognizing the newly-created RE site, reducing the presence of full-length normal sequences in the sample. Next, further PCR using the "screening primer" of the secondary PCR step is carried out, followed by restriction digestion, to enrich the sample for mutant alleles, and the full-length mutant bands are gel-purified. The digestion-resistant band indicates the presence

of a mutant allele, but does not define the sequence of a mutant allele. To demonstrate that a mutant allele was present originally, the gel-purified band is further amplified and digested using the same "screening" primer and restriction enzyme as used in the secondary PCR step (i.e., "enriched screening"). A digestion-resistant band indicates that a mutant allele was present in the starting sample, but does not identify the specific mutant present. To define the mutation, PCR is performed on the gel-purified product using mismatched "verification" primers that introduce new restriction sites into the PCR products derived from specific mutant alleles, the opposite of the screening strategy. The presence of a PCR product that cuts with the verification enzyme confirms that a specific mutation is present.

Nowhere does Jacobson teach using the ligase detection reaction in conjunction with its above-described procedure. Nowhere does Jacobson teach or suggest that the steps of the method described therein are useful or capable of being incorporated into another detection methodology.

The teachings of Barany and Jacobson are not properly combinable. As noted above, Barany teaches that single base differences in nucleic acid sequences can be effectively identified by use of a ligase detection reaction followed by the capture of ligation products on an addressable array. There is no indication that further procedures are desirable or even needed. The outstanding office action's suggestion that utilizing the features of Jacobson in conjunction with Barany's process would be beneficial in permitting the detection and identification of low abundance sequences is entirely speculative and based on nothing more than hindsight. Indeed, the method of Jacobson has been shown to be susceptible to a high degree of false positives, making it unsuitable for the purpose of the present invention, i.e., the detection and identification of low abundance sequences in a sample with a plurality of nucleotide sequences. The method of Jacobson, as noted in the instant application (page 7, line 9 to page 8 line 23) is a "primer-mediated" version of RFLP-PCR that uses PCR primers to introduce a new restriction site into the wild-type allele in the starting sample, followed by restriction digestion to remove the wild-type allele. However, subsequent investigators have demonstrated that errors are produced by polymerase extension from primers having 3' natural base mismatches such as those used in the method of Jacobson (e.g., Hattori et al., "Mismatch PCR RFLP Detection of DRD2 Ser311Cys Polymorphism and Schizophrenia," Biochem. Biophys. Res. Commun., 202(2):757-63 (1994) (PCR primers with mismatch at 3' end severely inhibited amplification at desired locus and produced unwanted product, while the PCR primer with a mismatch internal to 3' end amplified targets correctly without false

negatives or false positives; see Figure 1, Figure 2, and discussion pp. 761-762) (attached hereto as Exhibit 1); O'Dell et al., "PCR Induction of a TaqI Restriction Site at Any CpG Dinucleotide Using Two Mismatched Primers (CpG-PCR)," Genome Res., 6(6):558-68 (1996) (mispriming events created unwanted extension products when mismatch-PCR primer had C or G at 3' end; see pg. 562, para. bridging bottom col. 1-top col. 2) (attached hereto as Exhibit 2); and Hodanova et al., "Incorrect Assignment of N370S Mutation Status by Mismatched PCR/RFLP Method in Two Gaucher Patients," J. Inherit. Metab. Dis., 20(4):611-2 (1997) (failure of PCR/RFLP to detect the N370S mutation, while the genotype established by sequencing was N370S/D409H, was attributed to failed digestion by the restriction endonuclease; see pg. 611, para. 3-4) (attached hereto as Exhibit 3). Such templates fail to cleave during restriction digestion and amplify as false positives that are indistinguishable from true positive products extended from mutant templates. Given the exponential amplification that occurs with PCR, it is clear that even a few erroneous templates can produce a large population of false positive extension products. In view of the propensity of Jacobson's process to yield false positive results, one of ordinary skill in the art would have no motivation to utilize this procedure in conjunction with Barany's procedure for detecting nucleic acids with single base differences.

Even if Barany and Jacobson were properly combinable, which they are not, their combination would not teach the claimed invention. The claimed method sets forth an elaborate and specific series of steps. Merely asserting that the procedures of Barany and Jacobson are combinable does not obviate the need to demonstrate that these processes would have been combined in the manner which results in the specific series of steps claimed by applicants.

The rejection of dependent claim 17 should also be withdrawn. Claim 17 requires "blending the ligation product sequences and the restriction endonuclease, . . . thereby selectively destroying the high abundance tertiary extension products." Neither Barany nor Jacobson teach "blending the ligation product sequences and the restriction endonuclease" (let alone the prior steps of forming that mixture or providing the mixture's components). Nowhere does Barany teach carrying out a RE digest. Although Jacobson uses multiple RE steps, it does not use RE digestion in combination with LDR, nor is there any suggestion by Jacobson to combine a RE digest with, or following, a LDR step.

For all the above reasons, the rejection of claims 1, 3-7, and 17 under 35 U.S.C. § 103 should be withdrawn.

The rejection of claim 2 under 35 U.S.C. § 103(a) for obviousness over Barany in view of Jacobson, and further in view of Day et al., "Detection of Steroid 21-Hydroxylase Alleles Using Gene-Specific PCR and a Multiplexed Ligation Detection Reaction," Genomics, 29:152-62 (1995) ("Day") is respectfully traversed, because Day does not overcome the above-noted deficiencies of Barany and Jacobson.

The rejection of claims 8 and 10-16 under 35 U.S.C. § 103(a) for obviousness over Barany in view of Jacobson and further in view of U.S. Patent No. 5,859,221 to Cook et al. ("Cook") is respectfully traversed in view of the following comments.

Cook is cited as teaching nucleotide analogs which are resistant to nuclease degradation and exhibit hybridization properties of higher quality relative to wild-type DNA. Cook has nothing to do with a method of identifying one or more low abundance sequences differing by one or more single base changes, insertions, or deletions from a high abundance sequence by utilizing a ligase detection reaction, as in Barany. There is also no suggestion that Cook's modified oligonucleotides can be utilized in conjunction with an endonuclease digestion reaction, as disclosed by Jacobson. Accordingly, there would have been no reason to combine the teachings of Cook with those of Barany.

Even if Cook were properly combinable with Barany and Jacobson, which it is not, that combination would not teach the claimed invention, because this combination of references does not disclose "providing a pre-secondary oligonucleotide primer set" where at least one primer "contains one or more nucleotide analogs". The cited references also fail to teach "blending the primary extension products, the pre-secondary oligonucleotide primers, and the polymerase to form a pre-secondary polymerase chain reaction mixture" and "subjecting the pre-secondary polymerase chain reaction mixture to two or more polymerase chain reaction cycles".

Therefore, the obviousness rejection of claims 8 and 10-17 based on the combination of Barany, Jacobson, and Cook should be withdrawn.

The rejection of claim 9 under 35 U.S.C. § 103(a) for obviousness over Barany in view of Jacobson and Cook, and further in view of Day is respectfully traversed, because Day does not overcome the above-noted deficiencies of Barany, Jacobson, and Cook.

In view of the all of the foregoing, applicants submit that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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